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Eudragit Coating of Chitosan-Prednisolone Conjugate Microspheres and In Vitro Evaluation of Coated Microspheres

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Chitosan-prednisolone conjugate microspheres (Ch-SP-MS) were prepared, and Eudragit coating was applied in order to efficiently deliver the microspheres and drug to the intestinal disease sites. The Eudragit L100-coated microspheres (Ch-SP-MS/EuL100) were examined for particle characteristics and the release of drug and Ch-SP-MS in different pH media at 37°C. Ch-SP-MS were spherical, with a mean size of 4.5 μm and prednisolone content of 3.3% (w/w). Ch-SP-MS/EuL100 were fairly spherical, with a mean size of 22. 5 μm and drug content of 0.32% (w/w). At pH 1.2, the release extent was less than 5% even at 48 h, and Eudragit coating tended to suppress the release. In contrast, at pH 6.8 and 7.4, Ch-SP-MS/EuL100 tended to show somewhat faster drug release than Ch-SP-MS. Ch-SP-MS/EuL100 displayed a release extent of 23 and 27% at pH 6.8 and 7.4, respectively. Ch-SP-MS aggregated at pH 1.2, but almost kept their initial size and shape at pH 6.8 and 7.4. Ch-SP-MS/EuL100 almost maintained their original shape and size at pH 1.2, and gradually released Ch-SP-MS at pH 6.8 and 7.4 due to dissolution of the Eudragit layer. Eudragit coating is suggested to be useful to efficiently deliver Ch-SP-MS to the intestinal disease sites.

Keywords chitosan-prednisolone conjugate microspheres; Eudragit coating; particle characteristics; drug release; pH

INTRODUCTION

Ulcerative colitis and Crohn's disease are major forms of inflammatory bowel disease (IBD), and are crucial diseases because they are often severe, refractory and chronic (Danese & Fiocchi, 2006; Fiocchi, 2002). Recently, various chemical modifications (Anderson & Taphouse, 1981; Anderson et al., 1985; Mcleod et al., 1993; Mehvar et al., 2000) or drug delivery systems (Lamprecht et al., 2001a; Nakase et al., 2000; Tozaki et al., 1999a; Tozaki et al., 1999b; Yano et al., 2002) have been developed in order to obtain a highly effective and low-toxic therapeutic system against such IBD. 5-Aminosalicylic acid (5-ASA), and steroidal and non-steroidal anti-inflammatory drugs are frequently chosen for the treatment of IBD (Gionchetti et al., 2003). However, when these drugs are administered in simple conventional oral dosage forms, they are absorbed systemically to a large extent and not delivered efficiently to the disease sites, resulting in less effectiveness and greater toxic side effects. Therefore, to achieve high effectiveness and low toxicity, it is necessary to deliver a drug specifically to the target site and supply the drug efficiently for a sufficient period. Salazosulfapyridine is a prodrug of 5-ASA, and releases 5-ASA in the colon based on reductive action by colonic bacteria. Although salazosulfapyridine is clinically available, sulfapyridine, regenerated from salazosulfapyridine, can cause toxic side effects due to the intestinal absorption of sulfapyridine (Klotz, 1985). A commercial tablet, Pentasa, is a delayed release system of 5-ASA around the colonic region, and is considered to be less toxic than salazosulfapyridine because it releases only 5-ASA (Larouche et al., 1995; Onishi et al., 2005). Chitosan capsules containing 5-ASA have also been found to display a good effect as a result of their efficient biodegradation followed by the effective release of 5-ASA in the lower intestine (Tozaki et al., 2002).

As compared with 5-ASA, prednisolone (PD) is generally chosen for the treatment of more severe IBD. Although PD displays high efficacy at a low dose, it can induce severe toxic side effects such as immunosuppression (Yano et al., 2002); therefore, site-specific delivery of PD is considered to be important to obtain high efficacy and suppressed toxicity. Recently, micro- or nano-particulate dosage forms have been reported to be effective to deliver drugs to the intestine, Peyer's patches or colon (Lamprecht et al., 2001a; Lamprecht et al., 2001b; Nakase et al., 2000; Tabata et al., 1996; Van Der Lubben et al., 2001). Microparticles with a diameter larger than

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200 μm are not appropriate for long residence at the intestinal disease site due to their elimination by diarrhea (Lamprecht et al., 2001b; Watts et al., 1992), and nanoparticles and microparticles with a smaller size are superior for long residence at the site of colitis because they are trapped by a thick mucous layer (Lamprecht et al., 2000; Lamprecht et al., 2001b; Lamprecht et al., 2001c). Further, microparticles of several hundred nanometers to several micrometers are effectively taken up by macrophages appearing in large numbers (Onishi et al., 2005; Tabata et al., 1996; Van Der Lubben et al., 2001). Biocompatible and biodegradable polymers are expected to be useful to deliver drugs to inflamed areas, and may accelerate drug release due to their degradation by the enzymes of bacteria and macrophages (Tozaki et al., 1997; Tozaki et al., 2002); therefore, we developed novel chitosan-prednisolone conjugate microspheres as reported previously (Onishi et al., 2005). They behaved not only as a prodrug of PD but also as microspheres, and are suggested to be useful as a prolonged release system at disease sites. However, in order to deliver the drug and microspheres effectively to intestinal disease sites, it is necessary to avoid the effect of gastric pH and gastric mucoadhesion (Shimoda et al., 2001). Namely, enteric coating or enteric encapsulation is needed for practical application. In this study, the conjugate microspheres were coated with Eudragit, and the resultant coated micropsheres were evaluated in vitro by examining the particle characteristics, drug release and release behavior of initial conjugate microspheres in different pH media.

MATERIALS AND METHODS

Chemicals

Chitosan (Ch) (viscosity grade = 1000 (5 g/L, 20°C), deacetylation degree = 80% (mol/mol)), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) and liquid paraffin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Prednisolone (PD) and prednisolone 21-hemisuccinate (SP) sodium salt (SP·Na) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Eudragit L100 was obtained from Rohm GmbH Chemische Fabrik (Darmstadt, Germany). Sorbitan sesquioleate (SO-15) was purchased from Nikko Chemicals Co., Ltd. (Tokyo, Japan). All other chemicals used were of reagent grade.

Preparation of Conjuate Microspheres and their Eudragit Coating

Preparation of Conjugate Microspheres

First, chitosan-succinyl-prednisolone conjugate (Ch-SP) was prepared by modifying the reaction conditions stated in the previous report (Onishi et al., 2005). As compared with the previous conditions, in the present experiment, solvent volume was increased to reduce the viscosity of Ch solution, and the stirring rate was raised to heighten mixing efficiency. Further, in order to strengthen the reaction efficiency, the amount of EDCI was increased to some extent, and the reaction time was made longer to 48 h. Briefly, Ch (120 mg) was dissolved in water (85 mL) acidified by adjusting the pH to 5 with 1 M HCl aqueous solution, and 5 mL of water containing SP·Na (40 mg) was added. After the pH of the mixture was adjusted to pH 5–5.5 with 1 M NaOH aqueous solution, 200 mg of EDCI, dissolved in 5 mL water, was added, and stirred at 1000 rpm for the initial 5 h under the ice cooling and for the next 19 h at room temperature. Then, 5 mL of water containing 200 mg of EDCI was added, and stirred at 1000 rpm for 24 h at room temperature. The resultant solution was poured into a 4-fold volume of acetone to precipitate Ch-SP. The precipitate was suspended with a mixture of water and acetone (1:4, v/v), and centrifuged at 3000 rpm for 5 min to wash Ch-SP. This operation was repeated three times. The final precipitate was suspended in 60 mL of water, and lyophilized to Ch-SP powder. The proposed chemical structure of Ch-SP is described in Figure 1, in which the carboxy ester is considered to be a cleavable bond.

Ch-SP (50 mg) was dissolved in 1% (v/v) acetic acid aqueous solution, added to 150 mL of liquid paraffin containing SO-15 at 1% (w/v), stirred at 90°C and 1000 rpm for 1 h, and then at 1000 rpm and 95°C for 30 min. The temperature of the mixture was then returned to room temperature, and *n*-hexane was added. The mixture was centrifuged at 3000 rpm for 5 min to precipitate the product. The precipitate was washed three times by suspension in n-hexane and subsequent centrifugation. The final precipitate was obtained by filtration using a Teflon filter (pore size = 0.1 μm), and dried in a desiccator to obtain Ch-SP microspheres (Ch-SP-MS).

Eudragit Coating of Conjugate Microspheres

Eudragit L100 (200 mg), the structure of which is shown in Figure 1, was dissolved in 2 mL of methanol, and Ch-SP-MS (50 mg) were suspended in Eudragit solution. The suspension was added to 100 mL of liquid paraffin containing SO-15 at 1% (w/v), and stirred at 500 rpm and 50°C for the initial 1 h and at 500 rpm and 55°C for the next 30 min. Then, the mixture was cooled to room temperature, and *n*-hexane was added. The mixture was centrifuged at 3000 rpm for 5 min to precipitate the product, and the precipitate was washed three times by suspension in *n*-hexane and subsequent centrifugation. The final precipitate was obtained by filtration using a Teflon filter (pore size = 0.1 μm; Toyo Roshi Kaisha, Ltd., Japan), was dried in a desiccator to yield Eudragit-coated Ch-SP microspheres (Ch-SP-MS/EuL100).

Investigation of Particle Characteristics

The drug content of Ch-SP was determined by alkaline hydrolysis and subsequent measurement of the absorbance of the resultant supernatant as shown in the previous report (Onishi et al., 2005); that is, Ch-SP (2 mg) was placed in a 0.1 M NaOH aqueous solution, and the mixture was kept at 45°C for 20 min. After centrifugation of the mixture, the supernatant

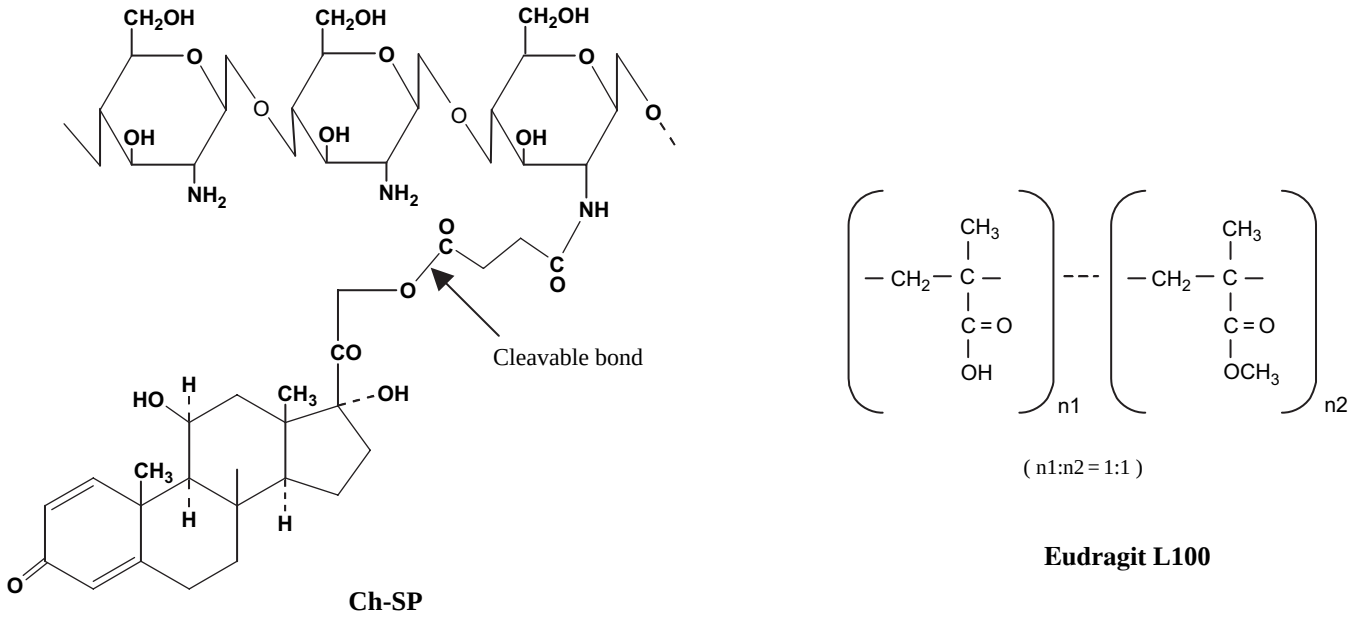

FIGURE 1. Chemical structures of chitosan-succinyl-prednisolone conjugate (Ch-SP) and Eudragit L100.

was measured spectrophotomtrically at 264 nm. The drug content of Ch-SP-MS and Ch-SP-MS/EuL100 was examined in a similar manner.

The particle shape and size of Ch-SP-MS and Ch-SP-MS/EuL100 were investigated based on scanning electron microscopy (SEM) for each powder prepared. The powder samples were thinly coated with platinum, and observed using a JEOL JSM-5600LV scanning electron microscope. The particle size and its distribution were examined by measuring the Green diameters of 80 particles chosen at random. The particle shape and surface were also investigated using the same SEM micrographs.

In Vitro Release Studies

Ch-SP, Ch-SP-MS and Ch-SP-MS/EuL100 were placed in 5 mL of JP14 1st fluid (pH 1.2), JP14 2nd fluid (pH 6.8) or phosphate-buffered saline (pH 7.4) (PBS) at a concentration of 50 μg PD eq/mL. Each mixture was incubated at 100 rpm in a water bath warmed to 37°C. At 1, 3, 7, 24, and 48 h after the start of the incubation, the mixture was centrifuged at 3000 rpm for 5 min, and the supernatant (100 μL) was withdrawn and stored in a freezer at −20°C until analysis. After each sampling, the incubation mixture was gently stirred, and the incubation was restarted. The time that the incubation mixture was immersed in the water bath at 37°C was regarded as the incubation time. Each sample was diluted to a 3-fold volume by addition of the mobile phase of high performance liquid chromatography (HPLC), and the resultant solution was measured for PD by HPLC.

HPLC Assay

The concentration of PD in the samples obtained in the in vitro release test was determined by HPLC. The HPLC system consisted of an LC-6AD pump, an SPD-10AV spetrophotometric detector and a C-R7A plus chromatopac (Shimadzu Corp., Kyoto, Japan). The detector was set at 246 nm. A Supelcosil LC-18-DB column (4.6 mm in inner diameter × 15 cm in length, 3 μm beads) (SUPERCO), used as an analytical column, was kept at 30°C by the column oven. A 22% (w/v) 2-propanol aqueous solution containing 0.1% (w/v) trifluoroacetic acid was used as the mobile phase, and the flow rate was 1.0 mL/min. Determination was performed by the absolute calibration curve method.

Examination of Particle Characteristics in Different pH

Ch-SP-MS and Ch-SP-MS/EuL100 were incubated under the same conditions as the in vitro release studies. At 1, 2, and 4 h after the start of incubation, a certain amount of suspension was withdrawn, and centrifuged at 3000 rpm for 5 min. The precipitate was washed three times with water, and dried in a desiccator. The resultant solid or powder was observed by SEM in the same manner as described above. The Green diameters of 80 particles chosen at random in each SEM micrograph were measured to obtain the mean diameter and size distribution of each sample.

RESULTS AND DISCUSSION

Particle Characteristics

The preparation of Ch-SP and Ch-SP-MS was performed modifying the reaction conditions described in the previous report (Onishi et al., 2005). The drug contents of intact and Eudragit-coated Ch-SP-MS are shown in Table 1. The PD content of Ch-SP was 7.7 ± 1.8 % (w/w), and that of Ch-SP-MS

TABLE 1
Particle Characteristics of CM-SP-MS and CM-SP-MS/EuL100

Formulation	Drug content[a] (%, w/w)	Particle size[b] (μm)	Size dirstribution[b] (min. – max., μm)
Ch-SP-MS	3.3 ± 0.7	4.5 ± 0.8	1.0 – 7.0
Ch-SP-MS/EuL 100	0.32 ± 0.07	22.5 ± 7.0	8.0 – 40.0

[a]The results are expressed as the mean ± *SD* (*n* = 3).
[b]The results are expressed as the mean ± *SD* (*n* = 80). The small particles on the surface were neglected for Ch-SP-MS/EuL 100.

was 3.3 ± 0.7 % (w/w). These results were similar to the previous results, but not identical. The change in reaction or preparation conditions affected the content of PD. Namely, the PD content of Ch-SP was greater than previously, which was considered to be due to the increase in EDCI and longer reaction time. For Ch-SP-MS, the stirring speed and evaporation conditions were slightly more stringent in order to reduce the particle size and completely evaporate water and acetic acid. As these changes could cause acceleration of hydrolysis of the ester bond, the PD content of Ch-SP-MS was considered to be slightly reduced as compared with previously. The PD content of Ch-SP-MS/EuL100 was approximately one-tenth of that of Ch-SP-MS. The addition of a fairly large amount of Eudragit L100 and loss of Ch-SP-MS in the preparation were considered to explain the PD content. The particle size and shape of Ch-SP-MS and Ch-SP-MS/EuL100 are shown in Figure 2. Ch-SP-MS were almost spherical, and Ch-SP-MS/EuL100 were somewhat spherical. Ch-SP-MS had a mean diameter of 4.5 μm, and the size of individual particles ranged from 1 to 7 μm, which was considered adequate for residence in the lower intestine and colon (Lamprecht et al., 2000; Lamprecht et al., 2001c; Tabata et al., 1996). The size of Ch-SP-MS/EuL100 was 4–5 times larger than that of Ch-SP-MS. In Ch-SP-MS/EuL100, Eudragit enclosed the assembly of Ch-SP-MS; however, the particle size of Ch-SP-MS/EuL100 varied to a fair extent (Table 1), which was considered to be dependent on the size of the assembly of Ch-SP-MS.

In Vitro Release

The drug release profiles were investigated using media of pH 1.2 (gastric pH), 6.8 (intestinal pH) and 7.4 (physiological pH) at 37°C (Figure 3). At pH 1.2, the drug release was very slow. In particular, the release rate was suppressed greatly in Ch-SP-MS/EuL100, which was considered to be due to the effect of Eudragit coating. The drug release rate was augmented with the increase of pH, suggesting that drug release was controlled by hydrolysis of the ester bond of the SP moiety (Anderson & Taphouse, 1981; Mcleod et al., 1993). Although the uptake of the incubation media by Ch-SP-MS was considered to influence the drug release, the release rate was found to be almost controlled by the process of hydrolysis of the ester of the SP moiety, because Ch-SP, quickly hydrated in aqueous media, showed a similar release rate as CP-SP-MS. The fact that the release of PD from Ch-SP-MS was completed rapidly in alkaline aqueous media also supported that the hydrolysis

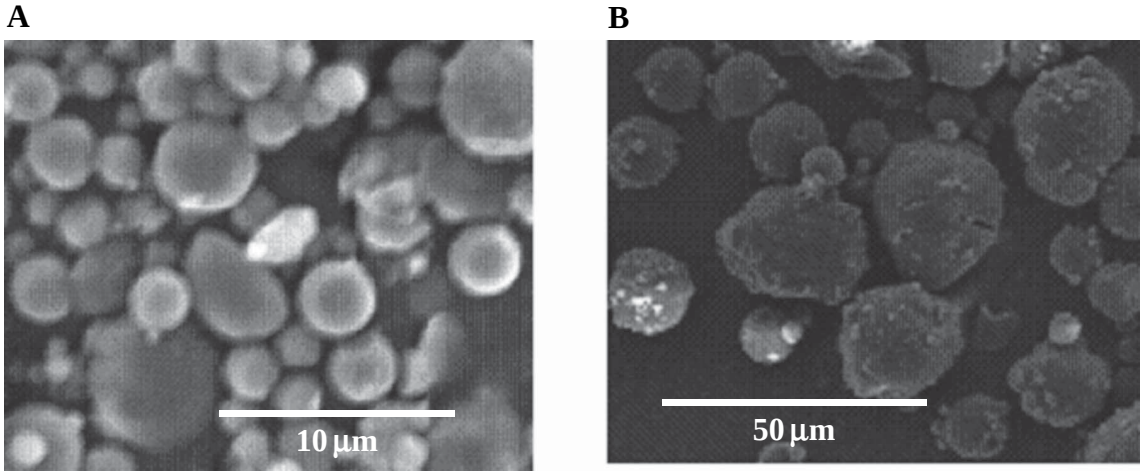

FIGURE 2. Scanning electron micrographs of Ch-SP-MS (A) and Ch-SP-MS/EuL100 (B).

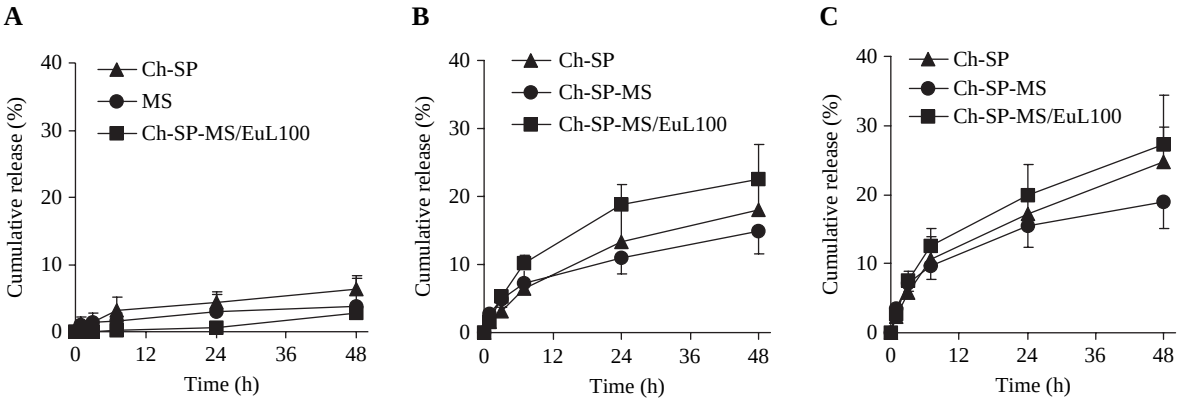

FIGURE 3. In vitro release profiles of PD from each formulation at 37°C in JP14 1st fluid, pH 1.2, (A), JP14 2nd fluid, pH 6.8, (B) and PBS, pH 7.4, (C). The results are expressed as the mean ± *SD* (*n* = 3).

of the ester would govern the release rate from Ch-SP-MS. At intestinal pH (pH 6.8), each formulation showed a gradual drug release. Ch-SP-MS/EuL100 exhibited a faster release rate than other formulations. The dissolution of Eudragit might facilitate the release of PD, that is, Eudragit molecules might affect chitosan chains and/or the ester bond of the SP moiety; though the details remained to be studied. At pH 7.4, the release rate was faster than at pH 6.8, which was considered to be due to the hydrolytic properties (chemical stability) of the carboxyl ester of the SP moiety. The 50% release time for Ch-SP, Ch-SP-MS and Ch-SP-MS/EuL100 at pH 7.4 was calculated to be 5.2, 7.4, and 4.6 days, respectively, when the pseudo-first order kinetics was applied. The release patterns were similar to those of pH 6.8. These results suggested that each formulation should suppress the release of PD at gastric pH and supply PD gradually at intestinal and physiological pH.

Change in Particle Features in Different pH Media

Although gradual drug release was achieved at intestinal pH for each formulation, interaction of the formulation with the gastrointestinal tract critically influences its transition (Sakuma et al., 1999; Shimoda et al., 2001). When simple Ch microspheres were administered orally, they were first trapped by the gastric mucosa, collapsed and/or dissolved there (Shimoda et al., 2001); therefore, protection of Ch microspheres from the effect of gastric pH and mucosa is needed. One of the methods is enteric coating of the micropsheres, and the effect of Eudragit coating on the particle status at different pH was investigated. The particle shape and size were monitored during incubation using the same media of pH 1.2, 6.8, and 7.4 as in the in vitro release studies. When the size distribution of Ch-SP-MS was examined by the dynamic light scattering method, some aggregation was found out by the comparison with the results of SEM micrographs (data not shown), suggesting that this light scattering method should be unsuitable for the determination of particle features. This is the reason why SEM micrographs were applied to the study of morphological change of the microparticles in the incubation media. The centrifugation, rinsing with water and drying might influence the morphologies of the microparticles in the incubation media. However, when the microspheres prepared with FITC-labeled Ch and their Eudragit-coated microparticles dispersed in the media were observed with the fluorescence microscopy, the results were consistent in size and shape with the results of SEM micrographs (data not shown), suggesting that the morphological features obtained by SEM should reflect those in the media. The process of brief washing and drying were considered to little influence the states of the remaining particles. Thus, SEM was applied for the analysis of morphological change of the microparticles in the media. As a result, Ch-SP-MS swelled, partly dissolved, partly collapsed and aggregated at pH 1.2, but almost kept the original size at pH 6.8 and 7.4 (Figure 4). On the other hand, Ch-SP-MS/EuL100 maintained their original shape and size at pH 1.2, which was considered to be due to the Eudragit coating. At pH 6.8 and 7.4, while Ch-SP-MS/EuL100 dissolved gradually, Ch-SP-MS were exposed progressively. After incubation of Ch-SP-MS/EuL100 at pH 6.8 and 7.4 for 4 h, Ch-SP-MS were almost exposed with the dissolution of Eudragit (Figure 4). The time courses of the particle size of Ch-SP-MS/EuL100 exposed in different pH media are shown in Figure 5. This indicated that Ch-SP-MS/EuL100 hardly exhibited the morphological change at gastric pH, and Ch-SP-MS were completely regenerated at intestinal pH several hours later as a consequence of dissolution of the Eudragit L100 coating layer. At that time, Eudragit coating appeared to protect Ch-SP-MS effectively at gastric pH because the drug release was suppressed by the coating (Figure 3). These features are suggested to be appropriate for the efficient delivery of Ch-SP-MS to disease sites located in the lower intestine.

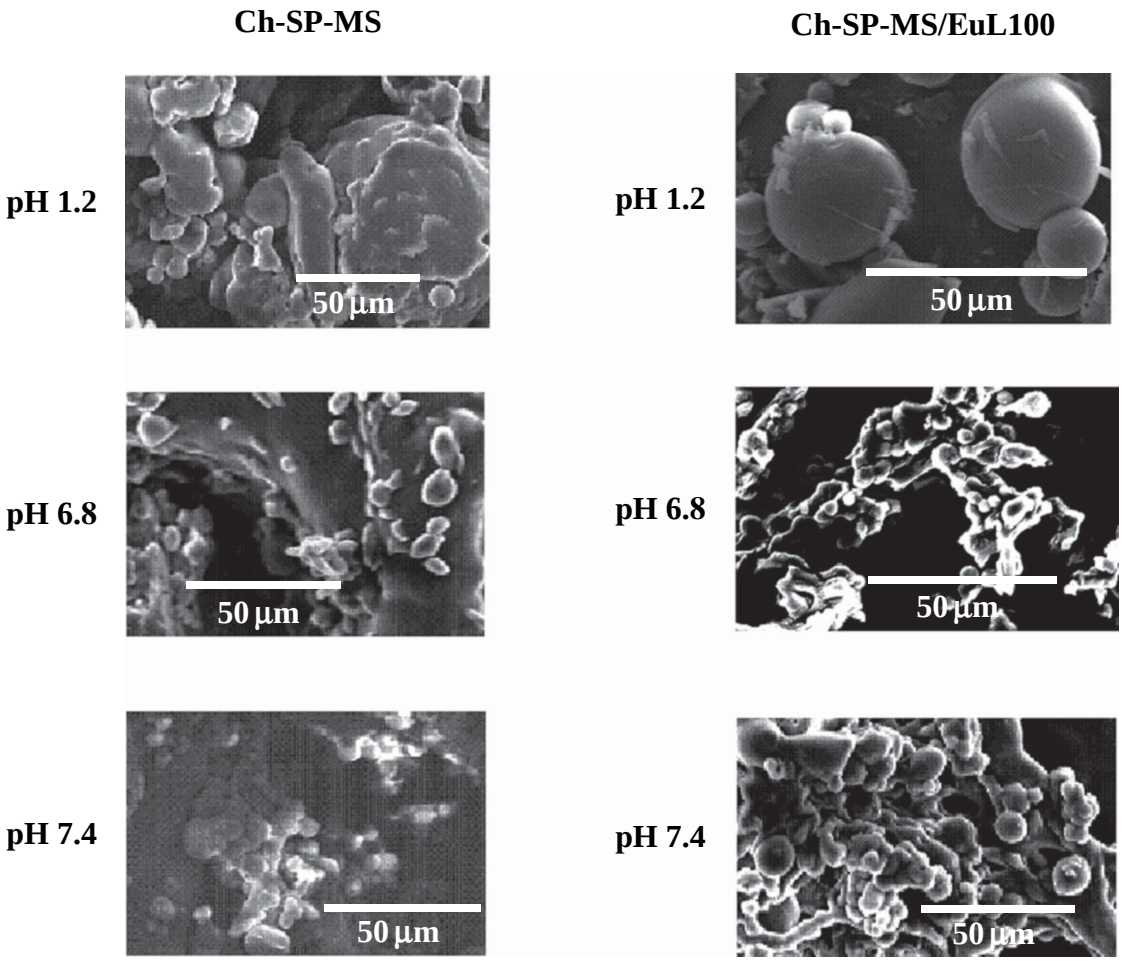

FIGURE 4. Particle states of Ch-SP-MS and Ch-SP-MS/EuL100 at 4 h after incubation at 37°C in the JP14 1st fluid, pH 1.2, JP14 2nd fluid, pH 6.8, and PBS, pH 7.4.

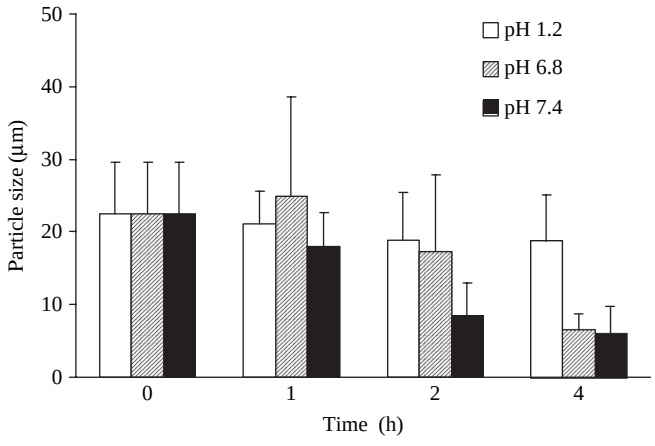

FIGURE 5. Change in particle size of Ch-SP-MS/EuL100 in the incubation at 37°C in the JP14 1st fluid, pH 1.2, JP14 2nd fluid, pH 6.8, and PBS, pH 7.4. The results are expressed as the mean ± *SD* (*n* = 80).

CONCLUSION

Conjugate microspheres (Ch-SP-MS) were coated with Eudragit. Non-coated and Eudragit-coated Ch-SP-MS were examined for drug release and particle characteristics in different pH media. PD was released gradually at intestinal pH, while the release was suppressed at gastric pH. Ch-SP-MS were protected from collapse or aggregation at gastric pH by Eudragit coating, and regenerated progressively at intestinal pH, suggesting that Eudragit-coated Ch-SP-MS should be useful as a delivery system of PD to the lower intestine. It may be needed for in vivo application or practical use to examine the coating extent and drug content in more detail.

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